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(71) Applicant: ELECTRO-NUCLEONICS, INC. [US/US]; 368 Passaic Avenue, Fairfield, NJ 07006 (US).

(72) Inventors: MANDLE, Richard, M.; 111 Watervliet Avenue, Pompton Lakes, NJ 07442 (US). WONG, Yuan, N.; 56 Intervale Road, Boonton, NJ 07005 (US).

(74) Agent: VASTA, Vincent, J., Jr.; Bryan & Bollo, Two Landmark Square, Stamford, CT 06901 (US).

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(54) Title: CHEMICAL LUMINESCENCE AMPLIFICATION SUBSTRATE SYSTEM FOR IMMUNOCHEMISTRY

(57) Abstract

A system for the detection of a biological analyte of interest which comprises contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an energy source which is capable of activating the fluorescer. A method for the qualitative and/or quantitative detection of a biological of interest is disclosed, which comprises: (1) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie; (b) contacting the fluorescer labeled specie and the biological of interest; (c) separating the fluorescer labeled specie/biological complex; (d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and (e) determining the presence of and/or measuring the quantum of chemiluminescent light emitted.

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CHEMICAL LUMINESCENCE AMPLIFICATION SUBSTRATE SYSTEM FOR IMMUNOCHEMISTRY

This invention relates to a system for the detection of a biological analyte of interest which comprises contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an energy source which is capable of activating the fluorescer.

Background Of The Invention

The clinician is concerned with detecting the presence of, and quantitatively measuring, a variety of substances via the use of many different analytical techniques. The most commonly used techniques employ absorbtiometry, both at visible and ultraviolet wavelengths, however, emission, flame photometry and radioactivity are also commonly used. A novel technique, thus far relatively unexplored in chemistry, is that employing the phenomenon of luminescence.

Analyses based on the measurement of emitted light offer several distinct advantages over conventionally employed techniques, including high sensitivity, wide linear range, low cost per test, and relatively simply and inexpensive equipment.

It has been predicted that the phenomenon of luminescence, and more particularly chemiluminescence, could have a major impact in two main aras of clinial analysis. First, it may have an important role as a replacement for conventional colorimetric or spectrophotometric indicator reactions in assays for substrates of oxidases and dehydrogenases. In this type of assay the sensitivity of the luminescence indicator reaction may be used to quantitate substrates not easily measured by conventional techniques (e.g., prostaglandins and vitamins).

The second major clinical application of luminescense might be in the utilization of luminescent molecules as replacements for radioactive or enzyme labels in immunoassay.

In each of these major clinical application areas, chemiluminescent reactions can provide a means to achieve a high level of analytical sensitivity.

Chemiluminescence may be simply defined as the chemical production of light. In the literature it is often confused with fluorescence. The difference between these two phenomena lies in the source of the energy which promotes molecules to an excited state. In chemiluminescence this source is the energy yielded as the result of a chemical reaction. The subsequent decay of molecules from the excited state back to the ground state is accompanied by emission of light, which is called luminescence. In contrast, in fluorescence, incident radiation is the source of the energy which promotes molecules to an excited state.

From an analytical point of view, the types of luminescence that have engendered the most interest are chemiluminescence and bioluminescence. The latter being the name given to a special form of chemiluminescence found in biological systems, in which a catalytic protein increases the efficiency of the luminescent reaction. Bioluminescent reactions such as the enzymatic firefly process, have been very useful analytically and convert chemical energy to light with a quantum efficiency of 88%.

In contrast to bioluminescence with the longevity and efficiency of the firefly, the history of chemiluminescence (hereinafter referred to as CL), especially that occurring in the non-aqueous phase, is remarkably short. The important aqueous CL substances luminol and lucigenin were discovered in 1928 and 1935,

respectively. A series of organic soluble CL materials were developed in the early 1960's based upon a study of the luminescent reactions of a number of oxalate compounds. A typical organic system useful for CL was disclosed by Bollyka et al., United States Patent No. 3,597,362, and claimed to exhibit a quantum efficiency of about 23% compared with about 3% for the best known available aqueous systems.

Chemiluminescence has become increasingly attractive for its potential in the clinical laboratory, especially for use in the analysis of a number of biologically associated materials, and its known applications have been the subject of thorough reviews, see for example: Whitehead et al. (1979) Analytical Luminescence: Its Potential In The Clinical Laboratory, Clin. Chem., 25, 9 1531-1546; Gorus et al. (1979) Applications Of Bio- And Chemiluminescence In The Clinical Laboratory, Clin. Chem., 25, 4 512-519; Isacsson et al. (1974) Chemiluminescence In Analytical Chemistry, Analytical Chemica Acta, 68, 339-362.

With few exceptions, most published CL clinical analytical applications have made use of the less efficient but well known diacylhydrazides, acridinium salts, pyrogallol, or lophine structures. It is important to appreciate that due to the nature of the chemical decomposition of the above chemiluminescent structures in the presence of hydrogen-peroxide, or generators of H_2O_2, as compared to that of the oxidation reaction of diaryl-oxalate structures, the latter has over 20 times the quantum yield of chemiluminescence, although its requirement for hydrogen peroxide is greater than the former.

Hydrogen peroxide, an essential component in the chemiluminescent reaction, has usually been the species selected for use in detecting the analyte of interest. For example, in the determination of glucose - Auses et al. (1975), Chemiluminescent Enzyme Method For Glucose. Analytical Chemistry, 47, No. 2, 244-248 employed the oxidation of glucose in the presence of glucose oxidase as the source of H_2O_2 which, in turn, was reacted with luminol to produce chemiluminescence in proportion to the initial glucose concentration. A limit of detection of 8×10^{-9}M peroxide was obtained with this system. Williams et al. (1976), Evaluation Of Peroxyoxalate Chemiluminescence For Determination Of Enzyme Generated Peroxide. Anal. Chem., 48, 7 1003-1006 in a similar reaction concluded the limit of sensitivity of the peroxyoxalate system is an order of magnitude poorer than that of the luminol system.

Therefore, until now the oxalic ester system (oxalate system) was generally thought to have little utility for analytical purposes due to its inefficient conversion of hydrogen peroxide.

The present invention overcomes this deficiency of H_2O_2 dependence by making use of the large chemiluminescent reservoir of energy in the oxalate system's chemistry. By using a suitable quantity of hydrogen peroxide and oxalate, a vast amount of energy may be concentrated in a form which is then released as chemiluminescence upon the introduction of a conjugated fluorescer.

Thus, the oxalate, acting in a fashion which can be visualized as analogous to a charged chemical battery,

releas s the stored energy to the fluorescer-conjugate in the same manner as an electrical switch in a circuit releases the energy of a battery to a lamp. This "switch" action causes chemiluminescence and, by conjugating the fluorescer to a detector of the analyte of interest, one can employ the reaction to trigger a detection system both qualitatively and quantitatively related to the analyte to be measured.

It is, therefore, an object of the present invention to provide for a system for the detection of a biological analyte of interest comprising contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an energy source which is capable of activating the fluorescer.

A further object of the present invention is to provide for a qualitative method for the detection of a biological analyte of interest comprising:

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie;

(b) contacting the fluorescer labeled specie and the biological of interest;

(c) separating the fluorescer labeled specie/ biological complex;

(d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and

(d) determining the presence or absence of chemiluminescent light emitted from the activated fluorescer.

A further object of the present invention is to provide for a quantitative method for measuring the amount of a biological analyte of interest comprising

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie;

(b) contacting the fluorescer labeled specie and the biological of interest;

(c) separating the fluorescer labeled specie/biological complex;

(d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and

(e) determining the amount of chemiluminescent light emitted from the activated fluorescer using appropriate instrumentation.

A further object of the present invention is to provide for a novel class of fluorescer materials which may be conjugated to an immunological specie specific to a biological of interest in order to provide for the detection of such biological.

A further object of the present invention is to provide for a novel class of conjugated fluorescer/biological compositions useful in the detection of various biologicals of interest.

Summary Of Invention

According to the present invention, there is provided a system for the detection of a biological analyte of interest comprising contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an energy source which is capable of activating the fluorescer.

There is also provided a method for the qualitative and/or quantitative method for the detection of a biological of interest comprising:

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie;

(b) contacting the fluorescer labeled specie and the biological of interest;

(c) separating the fluorescer labeled specie/biological complex;

(d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and

(e) detecting the presence of and/or measuring the quantum of chemiluminescent light emitted.

Additionally, there is provided for novel fluorescer and conjugated fluorescer/immunological specie compositions useful in the detection of various biological analytes of interest.

With respect to Charts I, II, and III, Rauhut

et al. (1969), Chemiluminescence Fr m Concerted Peroxide Decomposition Reactions, Accounts of Chemical Research, Vol. 2, 80-87, it can be seen that one mole of H_2O_2 is necessary to convert one mole of luminol into one mole of the energized or excited molecule. This exicted molecule then reverts to its ground state and emits light. Of interest is the fact that the CL compound, in Chart I, luminol or its derivatives, is also capable of converting the chemical energy of the system to light. Thus, the luminol acts as a source of CL energy and also as a fluorescer to absorb the energy and produce visible light. The luminol system is, therefore, not particularly useful in the context of the present invention since no differentiation between the light emitted upon fluorescer addition and that generated by the luminol itself can be made.

Charts II and III illustrate the fact that for the oxalate system, hydrogen peroxide does not always produce a species which gives rise to an excited state producing light. Some peroxide may be lost in side reactions which are "dark", thus, there is no predictable stoichiometric relationship between the H_2O_2 consumption and the quanta of emitted light.

Chart I

3-Aminophthalhydrazide Chemiluminescence in Reaction with Potassium Persulfate and Hydrogen Peroxide (Luminol)

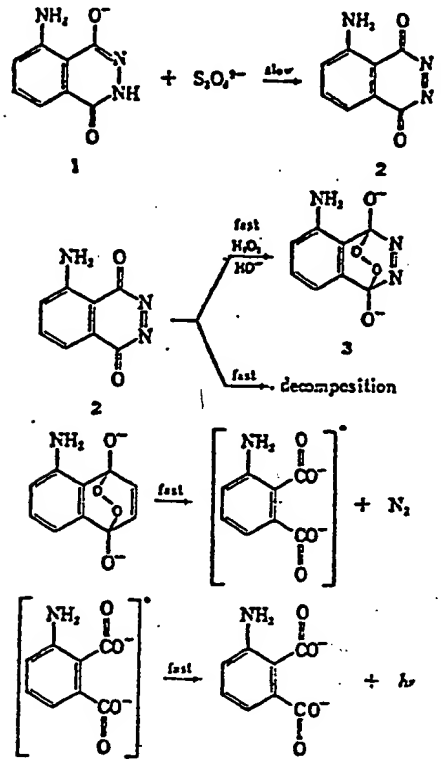


Chart II

Tentative Mechanism For Oxalyl Chloride Chemiluminescence

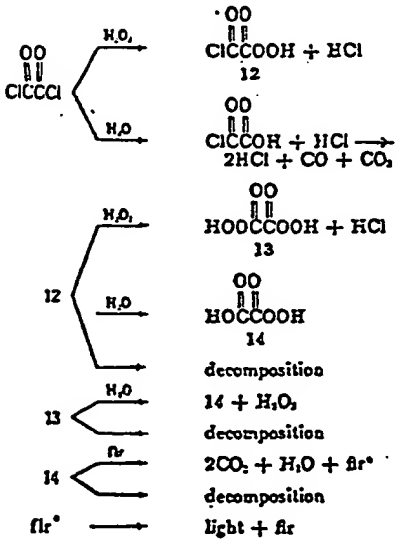
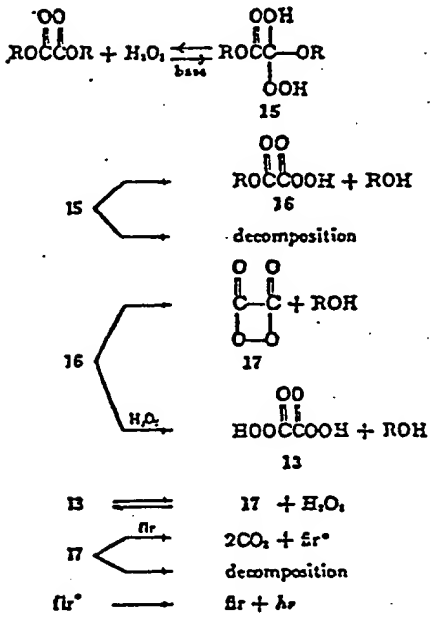


Chart III

Tentative Mechanism For Oxalic Ester Chemiluminescence



A major difference between the luminol system, which has been used to detect the presence or the quantity of H_2O_2, and the oxalate system is the requirement that the oxalates have an additional fluorescer to absorb the chemical energy generated in the reaction and then convert that energy to visible light. If the specified fluorescer is absent, the energy generated by the reaction will be dissipated without emitting visible light. The oxalate system is generally employed in an organic solvent and this requirement also has made its use in CL analytical methods less desirable than other CL materials, which are soluble in an aqueous medium, due to the incompatibility of biological anti-analytes to such organic solvents.

The present invention dramatically differs from the prior art utilizing CL for analytical purposes in the way the generated CL energy is employed. The present invention makes use of the CL system as a substrate or reservoir of chemical energy which emits light upon the addition of another compound, i.e. the fluorescer. We have found that by conjugating this fluorescer compound to the anti-analyte of interest it is possible to quantify the analyte's concentration in terms of the amount of emitted light. CL as thus applied becomes competitive as a highly sensitive replacement for radioimmunoassay techniques (RIA).

The comparison of Table 1 shows various analytical systems employing CL and illustrates the manner in which components of different reactions may be used to achieve detection. An analyte may be determined using CL by coupling the detector for the analyte to either:

I. A catalyst for generation of the H_2O_2 CL reaction, such as glucose-oxidase, or

II. A CL compound which generates CL energy and itself emits light, such as luminol, or

III. A fluorescer which absorbs chemical energy and emits light, such as a perylene derivative.

In each case, for the purpose of simplicity in this comparison, the analyte is assumed to be surface antigen to Hepatitis B (HB_SA_g) in human serum and is determined by a solid phase "sandwich" technique. This system is presently widely used with I^{125}, a radioactive isotope, as the label or indicator.

TABLE I

COMPARISON OF METHODS FOR USING COMPONENTS OF C.L. REACTION[1]

	Method I Conjugation of Oxidizer	Method II Conjugation of Luminol	Method III Conjugation of Fluorescer
Object of of Detection	H_2O_2	luminol	Fluorescer conjugate
Label example	glucose oxidase	luminol and derivatives	3,4,9,10-perylene tetracarboxylic dianhydride
Analogous Systems	Williams[2] (1976), Puget[3] (1977) Velan[4] (1978), McCapra[5] (1977)	Hersh[6] (1979), Pratt[7] (1978) Simpson[8] (1979), Schroeder[9] (1979) Olsson[10] (1979)	None
Advantages	1) Enzyme catalyst amplification system for hydrogen peroxide. 2) System can provide a number of readings before destruction. 3) Highest sensitivity for detecting H_2O_2.	None	1) Immunological reaction separate and distinct from CL production. 2) Highest level of light intensity, 23%. 3) Sample may be retested with additional oxalic ester. 4) Label is stable for coupling and storage. 5) Most desirable functional group may be used for attachment to biological material, minimizing destruction of label, specificity and activity. 6) Fluorescer inexpensive compared with enzyme. 7) Minimal or no instrumentation required for presumptive qualitative analyses.
Disadvantages	1) Many interfering substances also effect luminol reaction and light intensity. 2) Enzyme labeling, reactivity, and kinetics poor. 3) Even with high H_2O_2 levels quantum light low 1-3% max. 4) Sophisticated instrumentation required 5) Enzyme label expensive.	1) CL label consumed in reaction, thus sample destroyed. 2) Poor quantum yield of light, less than 1%. 3) Reaction susceptible to other catalysts and quenchers. 4) Sophisticated flow all instruments required.	1) Oxalate not generally available. 2) Extreme sensitivity of system may cause light emission from traces of foreign fluorescent materials.

Footnotes to Table 1

1 No solid phase system incorporating the advantages of a separation of CL, enzyme amplification and immunological chemistry has appeared in literature as described here.

2 Williams et al. (1976) Evaluation of Peroxyoxalate, Chemiluminescence for Determination of Enzyme Generated Peroxide, Anal. Chem., 48, 1003-1006

3 Puget et al. (1977) Light Emission Techniques For The Microestimation Of Femtogram Levels Of Peroxidase. Anal. Biochem., 79, 447-456

4 Velan et al. (1978) Chemiluminescence Immunoassay A New Method FOr Determination Of Antigens. Immunochemistry, 15, 331-333

5 McCapra et al. (1977) Assay Method Utilizing Chemiluminescence. British Patent No. 1,461,877

6 Hersh et al. (1979) Luminol-Assisted, Competitive-Binding Immunoassay Of Human Immuno-Globulin G. Anal. Biochem., 93, 267-271

7 Pratt et al. (1978) Chemiluminescence-Linked Immunoassay. Journal of Immunological Methods, 21, 179-184

8 Simpson et al. (1979) A Stable Chemiluminescent-Labelled Antibody For Immunological Assays. Nature, 279, 646-647

9 Schroeder et al. (1979) Immunoassay For Serum Throxine Monitored By Chemiluminescence. Journal Of Immunological Methods, 25, 275-282.

10 Olsson et al. (1979) Luminescence Immunoassay (LIA) A Solid Phase Immunoassay Monitored By Chemiluminescence. Journal of Immunological Methods, 25, 127-135.

In order to detect the antigen-antibody reaction the indicator in all cases illustrated in the comparison of Table 1 is taken to be the emission of light from CL. In the "sandwich technique" the following steps are taken: anti-HB_S (Goat) is coated to controlled pore glass (CPG) particles in tablet form (solid phase). Patient serum is added to a tube containing a CPG tablet. During incubation the tablet disintegrates. If Hepatitis B Surface Antigen is present in the serum tested, it will combine with the antibody on the glass particles. After incubation, the serum is removed and the glass beads rinsed. A label, as discussed below, conjugated to an anti-body specific to HB_SAg is then added. The labeled antibody combines with the antigen bound to the antibody on the glass particles forming the "sandwich". The labeled antibody then reacts in a specified manner in the CL system to give light as an indication of antigen presence. This CL assay is a qualitative test for the presence of Hepatitis B Surface Antigen in serum. In general, however, the greater the amount of HB_SAg in a sample, the greater the intensity of emitted light.

The reaction sequence and procedures used in carrying out the Methods illustrated in Table 1 were as follows:

Method I - Enzyme Chemiluminescent Immunoassay

Label: Antibody to Hepatitis B Surface Antigen conjugated with glucose-oxidase (GLO).

Reaction: (1) Glass.ab.ag + ab.GLO + glucose → H_2O_2

(2) Luminol + NaOH + H_2O_2 (from reaction 1) → light

Procedure: After incubation of the oxidase label to form the "sandwich" as described above, the complex is washed to remove excess label. The washed complex is then incubated for a fixed time with a standard glucose solution to allow the glucose substrate to form H_2O_2, the quantity of which is proportional to the original GLO present in the sandwich. An aliquot of the solution is then added to a standard catalyzed alkaline luminol solution with the light emission proportional to the HB_SAg in the original sample.

Method II - Chemiluminescent-labeled Immunoassay

Label: Antibody to Hepatitis B Surface Antigen labeled with luminol.

Reaction: (1) Glass.ab.ag.ab.luminol + H_2O_2 + hemin → light

Procedure: After incubation of the luminol label to form the "sandwich" as described above, the complex is washed to remove excess label. To the washed complex is added a standard hydrogen peroxide alkaline hemin reagent. The light emission is proportional to the HB_SAg in the original sample. It is noteworthy that Hersh et al. (1979) Luminol-Assisted, Competitive-Binding Immuno-Assay Of Human Immuno-Globulin, G. Anal. Biochem., 93, 267-271, end their paper describing a similar use of luminol with the following summary:

> "The luminol-based chemiluminescent label can be employed as a substitute for radiolabels in immunoassay for serum components at concentrations greater than 10^{-9} mol/liter. The main factor limiting the sensitivity of the method is the relatively low overal chemiluminescent efficiency (CE) of the

luminol tag. The CE of underivatized luminol is reported to be 1.5% (5). Our luminol-IgG label had a final efficiency of about 0.3%. It is possible that a more efficient means of coupling luminol, if found, would increase sensitivity by a maximum of 600%. The most efficient chemiluminescent system reported to date (not involving enzymes) is the hydrogen peroxide-oxalate ester reaction (6). This reaction has an overall chemiluminescence efficiency of 23%. The use of the oxalate ester as a chemiluminescent label would provide the more substantial gain of 1500% compared to the luminol system."

Thus, while earlier investigators recognized the quantum efficiency of the oxalate system for CL, they, like others, never appreciated the most efficient way to use this oxalate as a source of energy, would be by controlling the "switch" and not the "source" of the energy.

Method III - Chemiluminescent Labeled Light Amplification System

(The method of the present invention. - "CLASSIC")

Label: Antibody to Hepatitis B Surface Antigen conjugated to a perylene derivative fluorescer.

Reaction: (1) Glass.ab.ag.ab. perylene + TCPO + H_2O_2 → light

Procedure: After incubation of the perylene label to form the "sandwich" as described above, the complex is washed to remove excess label. The "sandwich" is then washed with tertiary butanol to remove excess buffer salts.

Then an excess of bistrichlorophenyl oxalate and hydrogen peroxide in dimethylphthalate are added to cause the fluorescer conjugate to emit light. The light emission is proportional to the HB_SAg in the original sample. The light intensity may be measured qualitatively by eye, or quantitatively by using a photodiode in the same manner that a photomultiplier in proximity to a sodium iodide crystal responds to the photons released by the gamma rays from the I^{125} label.

Discussion Of Methods I, II and III

The use of an oxidizer conjugated to an antibody (Method I) is in reality an adaptation of the well-known enzyme-immunoassay systems of Syva Corporation (United States Patent No. 3,817,837) and Organon Company (United States Patent No. 3,654,090) but here using CL as a light indicator instead of a dye color change. We are not aware of an analogous system incorporating all the solid phase sequences suggested herein. Nonetheless, the detection limit of this method is governed by the ability of the oxidase enzyme conjugate to liberate sufficient H_2O_2 as in the above enzyme immunoassays. Some increase in detection level may be achieved by using CL because of the better sensitivity of CL vs. dye color change, this sensitivity does not however approach the detection level of the fluorescer conjugate of Method III.

In Method II a number of analysts have suggested labeling the analyte detector with a CL compound or derivative. This method is inferior to Methods I or III in that the amount of light emitted can never be more than the total energy content of the amount of CL compound

conjugated - i.e., luminol or oxalate. A further disadvantage in coupling the CL compound directly to the antibody, for example, is the loss in CL capacity of the conjugate and the continued loss of light as the compound is consumed in the reaction. Finally, the entire loss of the consumed CL compounds before test completion prevents the analyst from repeating or rechecking the sample's CL.

Method III, alternatively referred to as "CLASSIC", the method of the present invention, overcomes the inherent disadvantages of Methods I and II. With "CLASSIC" it is possible to achieve the highest order of activity and specificity of the analyte detector because one can carefully select the preferred attachment site on the biological to be labeled. It is also possible to design the linkage of an efficient and durable fluorescer to conjugate with the biological effectively at this site without damaging the biological. Damage in specificity and activity of biologicals from I^{125} labeling, and damage to enzymes by conjugation is well known and an accepted fact in the preparation of immunodiagnostic reagents. A fluorescent label of preferred utility in CL, by its very structure, must be stable to the oxidizing conditions of the oxalate reaction. This inertness augers well in making fluorescers a particularly efficient form of label for immunochemical analyses.

The various levels of sensitivity and variations in different types of amplification is evaluated in a 1976 review by G. Wisdom, Enzyme-immunoassay, Clin. Chem., 22, 1234-1255. These systems provide the amplification for enzyme labels since enzyme catalytic properties allow them to act as amplifiers, and many enzyme molecules can

catalyze the formation of more than 10^5 product molecules per minute.

To be suitable as a label, an enzyme must meet the several criteria set forth by Wisdom (1976) (supra) which are as follows:

(1) Available cheaply in high purity.
(2) High specific activity.
(3) Stable under assay and storage conditions.
(4) Soluble.
(5) Assay method that is simple, sensitive, rapid, and cheap.
(6) Absent from biological fluids.
(7) Substrate, inhibitors, and disturbing factors, absent from biological fluids.
(8) Capable of retaining activity while undergoing appropriate linkage reactions.
(9) Capable of inhibition or reactivation when antibody binds to the enzyme-hapten conjugate.
(10) Assay conditions compatible with hapten-antibody binding.

These specifications are easily met by fluorescent organic compounds which may be readily coupled as labels capable of absorbing the chemical energy from the oxalate "substrate". In addition, as has been shown by Rauhut, certain selected fluorescer structures are capable of catalyzing the peroxyoxalate reaction products, thus providing the type of amplification available with enzymes. Whether such amplification does in fact take place has been question by Hastings et al. (1976) Photochem., Photobiol., 23, 461.

The CL system of the present invention, "CLASSIC", also has certain advantages over fluorescent antibody techniques which make use of the ability of a fluorescent tag to emit light of a particular wave length when excited by radiant energy of a lower wave length. A number of clinical analyses which utilize fluorescent "probes" or tags have been described in a recent review by Soini (1979) Fluoroimmunoassay: Present Status And Key Problems. Clin. Chemistry, 25, 353-361. In general, the detection level, or sensitivity, of fluoroimmunoassay techniques is greater than enzyme immunoassay techniques and approaches the capability of radioimmunoassay systems.

The use of fluorescent probes to replace radioactive isotopes is hindered by the decreased sensitivity obtained with fluorescence. This is due, to a great extent, to the sample's or serum's own fluorescence. The intensity of this background is affected by many fluorescing compounds, such as protein which may be present in the sample, and which also increase scattering caused by the specimen.

Fluorescence methods are now extensively applied in immunology, mainly in fluorescence microscopy, for studying various types of tissues, cells, bacteria, viruses and so on. A number of fluorescent materials and procedures for coupling them to the above biologicals and haptens is well developed.

To take advantage of the full scope of this invention, special high intensity fluorescent molecules are required. These must be capable of biological coupling with protein, polysaccharide and hapten

substances, especially immunoglobulins - i.e., I_gG - and antigens without disturbing the specificity or activity of these biological materials.

Bellin (1968) Photophysical and Photochemical Effects of Dye Binding. Photochem. and Photobiol., 8, 383-342 and Porro et al. (1963 and 1965) Fluorescence And Absorption Spectra Of Biological Stains. Stain Technology, Vol. 38, and Fluorescence And Absorption Spectra Of Biological Dyes (II). Stain Technology, Vol. 40, No. 3, 173-175, respectively, have shown that there is a reduction in efficiency in the light output of fluorescers as a result of bonding or conjugation to proteins as compared to the output of these fluorescers in free solution. Our work has shown a similar loss in output, however, the energy efficiency of the oxalate system compensates for this loss. While this loss in light output effects all other known applications of conjugated fluorescers, the analytical method of the present invention requires a conjugate only during the biological antibody/antigen formation phase of the analysis. Procedures are well known for preparing a conjugate of a fluorescer in a manner which permits the conjugate to be subsequently separated at will by changing the pH, or other parameter, of the conjugate solution. It should also be noted that the immuno-chemical reaction of CLASSIC, Method III, may be carried out in the environment best suited for the optimum detection of the analyte of interest. After the label has been identified with the analyte one may then separate the label, the fluorescer, from the conjugate which allows the fluorescer to enter the solvent phase of the CL system to yield the maximum light efficiency.

In general, it is desirable to provide the high quantum efficiency of fluorescing aromatic and substituted hydrocarbons, heterocyclic compounds, dyestuffs, and metal chelates with the ease of conjugation to the biological now available for microscopy reagents. We have found that we can couple the fluorescer using known procedures currently accepted for use with the fluorescent conjugates such as set forth in Soini (1979) supra, the teachings of which are incorporated herein by reference.

The following Tables 2 and 3 from Soini (1979) supra, set forth data on various fluorescent materials which can be advantageously employed as labels in the environment of the present invention.

Table 2

Published Data on the Properties of Fluorochromes Used for Various Purposes, Including Decay Times (τ), Quantum Yields (Q), Excitation and Emission Wavelengths, Absorbances (ϵ), and the Sensitivities of Fluorescence to Polarity Changes in the Environment [a]

Fluorochrome	τ, ns	Q_f	Q_b	Exc. max., nm	Em. max., nm	Mol. absorp.	Sensitivity of fluorescence to changes	Reference(s)
FITC	4,5		0, 85	492	518	72 000	stable	50, 51, 52, 53
TRITC				518		48 000	stable	55–60
RBITC	3		0, 7	550	585	12 300	stable	55, 57, 58
RB 200SC	1		0, 04	530, 565	595, 710		stable	48, 49, 55
DNS-Cl	14	0, 2	0, 3	340	520–480	3 400	sensitive	31, 32, 42, 47
Fluoram	7	0, 00	0, 1	394	475	6 300	sensitive	46, 65
MDPF		0	0, 1	390	480	6 400	not	61–64
NBD-Cl				468	530	12 900	sensitive	44, 45
ANS (ANM)	16	0, 0	0, 9	385	471		sensitive	41, 29
NPM (PBA)	100	0, 0		340	392, 375		sensitive	37, 38, 39, 40
DACM	5	0, 0	0, 5	383–398	480–482	24 200	sensitive	29, 33, 34, 35
BIPM	1	0, 0	0, 5–0, 8	310–315	360	28 000	sensitive	29, 33
anthracene-ITC	29		0, 6	357	460	3 040	not	48
FAM	20		0, 2	362	462			29, 36

List of abbreviations for Tables 1 and 2: ANS, 1-anilino-8-naphthalenesulphonic acid; ANSC, 1-anilino-8-naphthalenesulphonyl-chloride; BIPM, *N*-(*p*-2-benzimidazolyl-phenyl)-maleimide; DACM, *N*-(7-dimethylamino-4-methyl-2-oxy-3-chromenyl-)-maleidme; DNS-Cl, dansylchloride, dimethylaminonaphthalene-5-sulphonylchloride; FAM, fluoroanthylmaleimide; FITC, fluorescein-isothiocyanate; Fluoram, fluorescamine, 4-phenylspiro-(furan-2(3*H*)-1-phthalan)-3,3-dione; MDPF, 2-methoxy-2,4-diphenyl-3(2*H*)-furanone; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3 diazole; NPM, *N*-(3-pyrene)-maleimide; PBA, pyrenebutyric acid; RBITC, rhodamine-B200-isothiocyanate; RB-200-SC, lissamine-rhodamine B200-sulphonylchloride; TNS, toluidinonaphthalenesulphonic acid; TRITC, tetramethylrhodamine-isothiocyanate.

[a] (Q_f = quantum yield of free fluorochrome, Q_b = quantum yield of fluorochrome bound to protein).

Table 3

Fluorescence Maxima and Detection Limits of Some Probe Conjugates Based on Measurements by the Authors[a]

Probe	Binding type	Fluorescence max. (nm) Excitation	Emission	Detection limit In a buffer	In serum (1/10)	Remarks
FITC-BSA	covalent	491	517	30 ng/mL	1000 ng/mL	Interference by scattering
-hIgG	covalent	491	517	40 ng/mL	1400 ng/mL	Interference by scattering
-thyroxine	covalent	490	515	1 pmol/mL	35 pM/mL	Interference by scattering
RBITC-BSA	covalent	552	572	100 ng/mL	430 ng/mL	Interference by scattering
DNS-BSA	covalent	360	514	100 ng/mL	7000 ng/mL	Interference by serum fluorescence
-hIgG	covalent	360	514			
-thyroxine	covalent	330	480	30 nmol/mL?	2100 nmol/mL	Interference by serum fluorescence
-cys-digoxin	covalent	358	533	140 pmol/mL	98 nmol/mL	Interference by serum fluorescence
Fluoram-BSA	covalent	393	465	1500 ng/mL	32000 ng/mL	Interference by serum fluorescence
-hIgG	covalent	393	465			
-thyroxine	covalent	395	480	25 pmol/mL	535 pmol/mL	Interference by scattering fluorescence
NPM-BSA	covalent	335	392	100 ng/mL	1500 ng/mL	Interference by scattering and fluorescence
-IgG	covalent	340	392	2000 ng/mL		[b]
ANS-BSA	noncovalent	385	470			
ANSC-BSA	covalent	380	470	10000 ng/mL		[c]
TNS-BSA	noncovalent	322, 360	429			[d]
NBD-BSA	covalent	468	526	4000 ng/mL	240 µg/mL	[e]

Footnotes to Table 3

[a] Measurement results have been obtained with some commonly used probes as conjugates of bovine serum albumin, IgG, thyroxine, and digoxin. Conjugation was by common methods described in the literature (47, 37, 46, 44). No attempt was made to optimize measurement in any way, this was done directly at emission maxima for bandwidths of 10 nm. No cut-off filters were used. It would probably have been possible to reduce the detection limits of some probes considerably by altering the slit-values and by adjusting the measurement wavelengths, and by using suitable cut-off filters. (The emission of fluorescein for example is usually measured at 540 nm, although the emission maximum occurs at 515 nm.) The fluorescence and detection limits for different probe-conjugates were measured with a Perkin-Elmer fluorescence spectrometer, Model MPF-2A. The detection limits were measured in the regions of excitation and emission maxima, and the values compared with the background fluorescence values of diluted serum at the same wavelengths and with the same instrument sensitivity.

[b] No reaction in IgG, SH-groups.

[c] Serum background, may bind to different proteins.

[d] Interference by protein fluorescence.

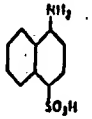
[e] Interference by serum fluorescence, own fluorescence weak.

BSA, bovine serum albumin; hIgG, human immunoglobulin G.

Typical of fluorescers which provide derivatives to which the biological may be coupled are the following from Pringsheim (1946) Luminescence Of Liquids And Solids And Its Practical Applications. Interscience Publishers, Inc., New York, New York, as Tables 4-6:

TABLE 4

Aromatic Hydrocarbons And Heterocyclic Compounds
(Neutral In Liquid Solution)

Name	Formula	Fluorescence Bands, Å	Excitation,* Å	Fluorescence Color	Page
1. Benzene		6 bands from 2600-3000	2600	U.V.	28, 100
2. Naphthalene		12 bands from 3000-3650	33000	U.V.	73, 100
3. α-Naphthol		3900-5600 with peak 4100	4000	blue	117
4. α-Naphthionic acid		4000-5600 with peak 4500 in alkaline solution	4000	blue green	117
5. α-Napthylamine		3900-5600 with peak 4100	4000	blue	73, 117
6. Anthracene (pure)		4 bands 3500-4550	3500	violet	28,73,100 et seq.
(commercial)		(naphthacene bands)	(4360)	(green)	109,131
7. Phenanthene (pure) (commercial)		8 bands 3460-4065 (anthracene bands)	3500	deep violet (blue)	102, 105

* Long wave-length limit of excitation.

TABLE 4 - Continued

Aromatic Hydrocarbons And Heterocyclic Compounds
(Neutral In Liquid Solution)

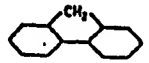
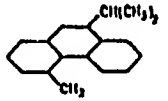
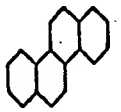
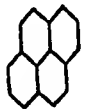
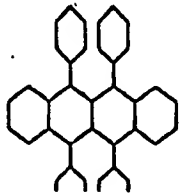
8. Fluorene (pure) (commercial)		3020-3700 (anthracene bands)	2940	U.V. (blue)	28, 103 153
9. Retene		3 bands 3400-3700		U.V.	102
10. Naphthacene		4 bands 4500-6500	4360	green	28,73,100 et seq. 109,131
11. Chrysene (pure) (commercial)		3 bands 3600-4000 (anthracene bands?)		deep violet (blue)	103, 131
12. Pyrene		5 narrow bands 3700-4000		U.V.	102
13. Rubrene		5300-6500 with peak 5900	5000	yellow	29, 73

TABLE 4 - Continued

Aromatic Hydrocarbons And Heterocyclic Compounds
(Neutral In Liquid Solution)

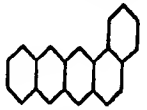
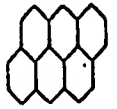
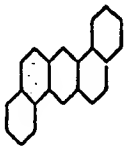
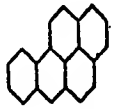
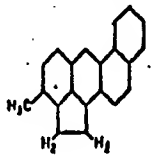
Name	Formula	Fluorescence Bands, Å	Excitation, Å	Fluorescence Color	Page
14. Pentacene		?		red	
15. Dibenzanthracene				yellow green	101
16. Anthanthrene				blue	73
17. Dibenzanthracene		3 bands 3900–4600		blue	101
18. Benzopyrene		4 band groups 4000–4320		blue violet	101
19. Methyl cholanthrene		broad band 4000–5200 with peak at 4600		greenish blue	102

TABLE 4 - Continued
Aromatic Hydrocarbons And Heterocyclic Compounds
(Neutral In Liquid Solution)

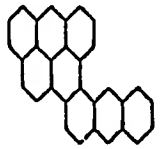
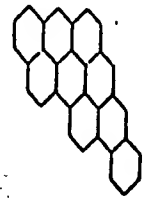
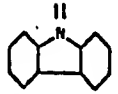
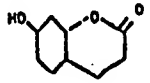
20. Perylene		4400–4700	violet	72
21. Pyranthrene			green	72
22. Violanthrene			green	72
23. Quinoline		3550–4900	blue	72
24. Acridine in acid solution		broad band 4000–4600 with 4 peaks broad band 4500–5500 with 4 peaks	blue green blue	72, 107 107
25. Carbazole		broad band 4000–4700 with two peaks	dark blue	72, 101
26. Umbelliferone			blue	105, 117

TABLE 4 - Concluded

Aromatic Hydrocarbons And Heterocyclic Compounds
(Neutral In Liquid Solution)

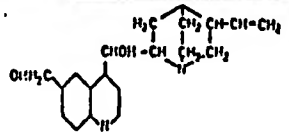
Name	Formula	Fluorescence Bands, Å	Excitation, Å	Fluorescence Color	Page
27. Quinine (in water)		4000-5000		violet	100, 115 et seq.
(in acid solution)		4200-6700 with two peaks 4600 and 5300		whitish blue	117 et seq., 132
28. Diphenyl-polyenes		Bands with 4 peaks			
n = 1		3300-4300		violet	72
n = 2		3600-4500		blue violet	
n = 3		4500-6300		sky blue	
n = 4		5000-7000		yellow green	
n = 5		6000		yellow	

TABLE 5

Synthetic Dyestuffs In Solution

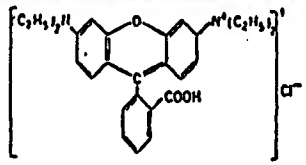
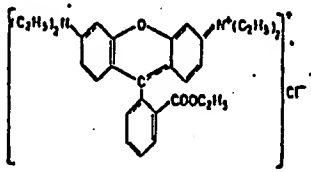
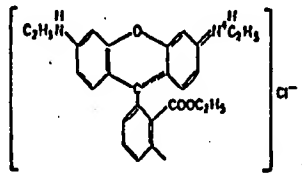
	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (of Peak), λ	Fluorescence: Color	Page
1.	749	Rhodamine B extra (416.5)		5500-7000 (6050)	red	28, 57 73, 122 127 et seq., 132
2.	751	Rhodamine 3 B (501.5)			orange	73, 135
3.	752	Rhodamine 6 G (450.5)		(5800)	yellow	73, 127 et seq., 132

TABLE 5 - Continued

Synthetic Dyestuffs In Solution

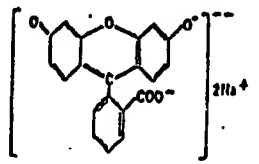
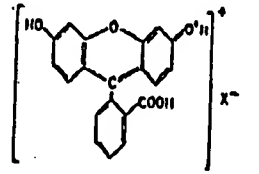
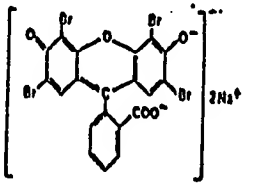
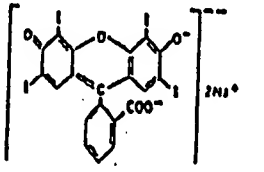
	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (of Peak), Å	Fluorescence: Color	Page
4.	766	Fluorescein in alkaline solution (370)		5000-6000 (5270)	yellow green	18, 25 67, 73 114, 127 et seq.
5.		Fluorescein in acid solution (370)		4500-5600 (4500)	blue green	114, 116
6.	765	Eosin G extra (692)		5100-6100 (5500)	yellow (5500)	23, 67 73, 110 123
7.	773	Erythrosin (830.4)		5000-6000 (5600)	yellow	28, 73 et seq. 129

TABLE 5 - Continued

Synthetic Dyestuffs In Solution

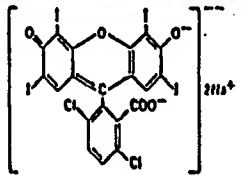
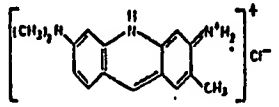
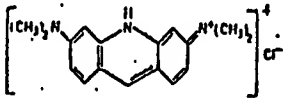
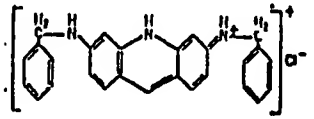
	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence		Page
				Wave-Length of Band (of Peak), Å	Color	
8.	779	Rose bengale ((ar2.5)		5400-6700 (6000)	yellow	73, 129
9.	787	Chloriphosphine O (287.5)			green	67, 73
10.	788	Euchrysine 3 R (Acridine orange) (301.5)		(6550)	yellow green	73
11.	789	Aurophosphine (457.5)			green	67

TABLE 5 - Continued
Synthetic Dyestuffs In Solution

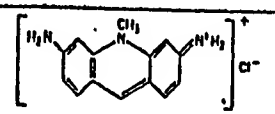
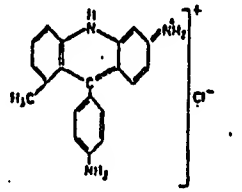
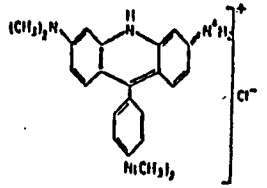
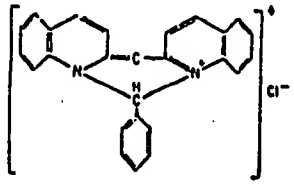
	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (of Peak), Å	Fluorescence: Color	Page
12.	790	Trypaflavine (259.5)		4600-5800 (5200)	yellow green	67, 73 110, 127
13.	791	Flavophosphine (333.5)			green	
14.	795	Rheonine A (fast phosphine) (365.5)			green	
15.	805	Auxin (quinoline red) (391.5)			orange red	

TABLE 5 - Continued

Synthetic Dyestuffs In Solution

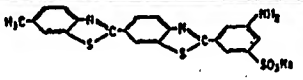
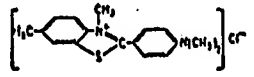
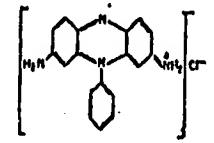
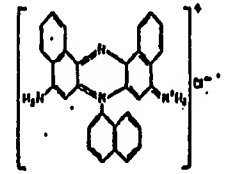
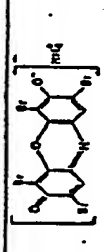
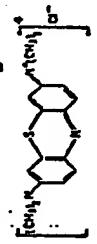
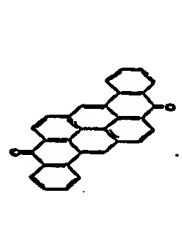
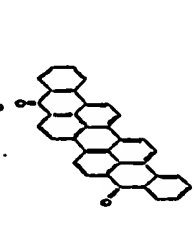
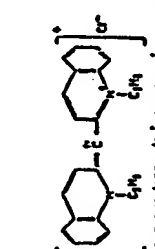
	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (of Peak), Å	Fluorescence: Color	Page
16.	812	Primulin yellow in acetone in water (475)			blue green (violet)	67, 73 127, 131
17.	815	Acridol yellow S (thioflavin S)			green	67, 127
18.	816	Rhodoline yellow (Thioflavin T) (318.5)			green	67, 73
19.	827	Safranine B extra (322.5)			yellow green	73
20.	848	Naphthyl blue (on silk)			red	
21.	857	Magdala red (478.5)		6500–6600 (5910)	orange red	73

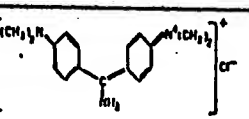
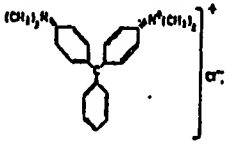
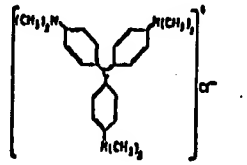
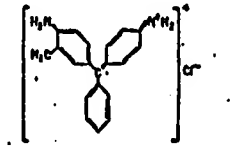
TABLE 5 - Continued
Synthetic Dyestuffs In Solution

	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wavelength of Band (of Peak), Å	Fluorescence: Color	Ref.
22.	908	Fluorescein blue (316)			red	23, 73
23.	922	Methylene blue (329.3)		(6700)	red	
24.	1066	Pyranthrene (indanthrene gold orange) (404)			green	73
25.	1079	Violanthrene B.S. (458)			red	73
26.	1253	Solanthrene jade green (on cloth)			green	
27.	1017	Pyranthrone-cyanine* (362)		narrow band 8720	yellow	

*Polymerized at high concentrations in water.

TABLE 5 - Continued

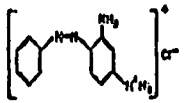
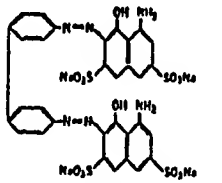
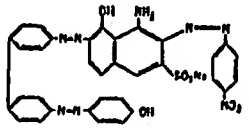
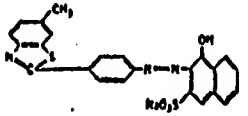
Synthetic Dyestuffs In Solution

	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (or Peak), Å	Fluorescence: Color	Page
28.	655	Auramine** (303.5)		4900-5900	green	73, 124 131
29.	657	Malachite green** (364.5)		4750-5600	green	73
30.	681	Crystal violet** (407.8)		6000-7600 (6700)	red	73
31.	731	Fuchsine** (322.8)		5550-6650	orange	

** In solid solutions or adsorbed on solid adsorbents.

TABLE 5 - Continued

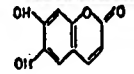
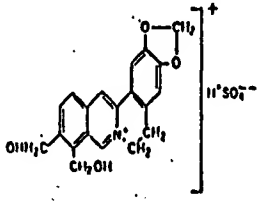
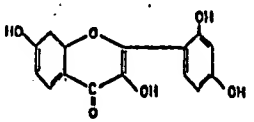
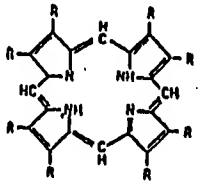
Synthetic Dyestuffs In Solution

	Colour Index No.	Name (Molecular Weight)	Formula	Fluorescence: Wave-Length of Band (of Peak), Å	Fluorescence: Color	Page
32.	27	Chrysoidine*** (248.3)			green	
33.	406	Direct sky blue***				
34.	520	Direct blue 2B*** (938)				74
35.	501	Diazil green† (710)			color varies with nature of cloth	74
36.	427	Ch rysamine G† (463)				74
37.	716	Benzaldine (III.)				74

*** When adsorbed on solid adsorbent
† When dyed on wool, silk, cotton or rayon

TABLE 6

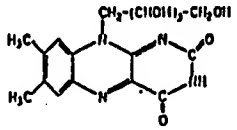
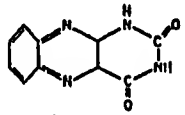
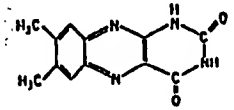
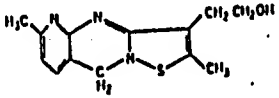
Natural Dyestuffs In Solution

	Formula	Fluorescence		Page
		Wave-Length of Band, λ	Color	
1. Aesculin		4000-5500 (4900)	blue	34, 57, 70, 117, 129
2. Berberine bisulfate			yellow green	67
3. (1231) Fisetin*			yellow	74
4. Porphyrins				
neutral or alkaline solution		series of narrow bands 5500-6000	orange red	28, 74
acidified solution		3 bands 6500-6600	orange yellow	103, 115

* Only when adsorbed on solid adsorbent.

TABLE 6 - Continued

Natural Dyestuffs In Solution

	Formula	Fluorescence		Page
		Wave-Length of Band, Å	Color	
6. (1299) Chlorophyll		3 bands 6300-8300	red	74, 102, 127
6. Urobilin (zinc complex)		3 bands 5150-6100**	green	103
7. Riboflavin (vitamin B_2)		symmetrical band 5000-6000 (5620)	greenish yellow	27,74,113 et seq., 115, 120
8. Alloxazine			blue	103, 113
9. Lumichrome 6,7-Dimethyl-alloxazine		4540-5470	sky blue	103, 115 120
10. Thiochrome (derived from thiamin or vitamin B_1)			sky blue	74, 104 112

** The band at 5200 A is by far the strongest.

In addition to the organic fluorescers listed above, a number f metal organic materials have been suggested for laser fluorescent assay systems: Ruthenium (II)-tri(bipyridyl) complex has been identified by Curtis et al. (1977), Chemiluminescence; A New Method For Detecting Fluorescent Compounds Separated By Thin Layer Chromatography, J. Chromatography, 134, 343-350 for CL applications; Metal Complexes by Sherman (1978), Analytical Applications Of Peroxyoxalate Chemiluminescence, Analytical Chim. Acta, 97, 21-27, and Soini (1979) supra. Weider United States Patent No. 4,058,732 disclosed and suggested their immunofluorescent application. It is also well known, Van Uitert (1960), Factors Influencing The Luminescent Emission States Of The Rare Earths. J. Electrochem. Soc., 107, 803, that small additions of the rare earth and/or transition metals function as promotors, activators or coactivators in inorganic and organic phosphors. Thus, it is not unexpected that trade impurities will behave in a similar manner in other organic and metallo-organic systems and have a profound effect on the quantum efficiency of the fluorescer.

The discussion has thus far centered around the novel analytical use of a fluorescer-biological conjugate activated by the chemical energy from a peroxyoxalate CL system. The preferred peroxyoxalate system is advantageous for CL because of its quantum efficiency and because there is no background light in the absence of a fluorescer conjugate. This system is particularly "noise free" when certain intensity control additives are eliminated, such as are disclosed by Bollyky (1972) Chemiluminescent Additives, United States Patent No. 3,704,231. A system for analytical purposes need only provide light

f high intensity for a sh rt period, that is, for example, under about 30 minutes.

While peroxyoxalates which are "noise free", or nonfluorescent are preferred, other naturally self-fluorescent oxalate esters or CL compounds are also useful with the proper selection of a barrier filter and use of a conjugate fluorescer of longer wavelength. Such esters include 2-napthol-3,6,8,-trisulfonic acid, 2-carboxyphenyl, 2-carboxy-6-hydroxyphenol, 1,4-dihydroxy-9, 10-diphenylanthracene, 2-napthol, as well as aqueous CL materials such as luminol, lophine, pyrogallol, luciferin, and related compounds.

Other systems besides those mentioned are also capable of activating a CL fluorescer-conjugate.

These include: (1) Ozone, which has been shown by Randhawa (1967), Ozonesonde For Rocket Flight, Nature, 213, 53, to activate Rhodamine-B. (2) Keszthelyi et al. (1969), Electrogenerated Chemiluminescence: Determination Of Absolute Luminescence Efficiency, etc., A. Chem., 47, 249-256, has demonstrated electrogenerated CL in 9,10-diphenylanthracene, thianthrene, and rubrene with some systems. Thus, Ozone or electro-generated CL in the presence of the fluorescer-conjugate can provide other useful energy sources for the CL fluorescer systems of the present invention. In addition, other known energy sources such as have been found useful in applications involving the distortion of various polymers by mechanical energy and other similar systems which yield free radicals are also useful in the present invention.

It should be understo d that many analytical system variations are possible, but all have in common the use of a labelled immunological specie specific to the analyte. The analyst has the latitude in selecting a procedure which provides the detection level required from a minimum amount of sample and which uses the least expensive and most reliable instrument. The detection level required is a function of the antigen, antibody or hapten concentration in the analyte and its clinical significance.

For clinically significant dosage testing, i.e. Digoxin, standard curves are obtained from known samples analyzed together with the unknown and run under carefully controlled duplicate analyses on highly calibrated instruments. While a presumative test for an immunoglogulin requires a much lower level of sophistication, it is highly advantageous for a single analytical system to be able to cover this analytical spectrum.

The sophisticated analytical requirements may be met by using a Centrifugal Fast Analyzer such as that made by Electro-Nucleonics, Inc. Burtis et al. (1975). Development Of A Multipurpose Optical System For Use With A Centrifugal Fast Analyzer. Clinical Chemistry, 21, 1225-1232. For the N^{th} nations lacking the ability or need for such sophistication, or for presumptive testing at the physician's office or clinic, no instrument is required. The "CLASSIC" system of the present invention delivers sufficient intensity to the labeled biological to enable the clinician to make a simple go-no-go determination by "eyeballing".

The clinician may also modify the role of the labeled specie used in carrying out the analyses. While solid phase techniques have been used as examples to illustrate the advantages of the present invention, it should be recognized that homogeneous and heterogeneous assays also will benefit from the use of the "CLASSIC" system. Acceptable alternative variations in test procedure include:

(1) Competitive binding of labeled antigen.
(2) Competitive binding of labeled antibody.
(3) Quenching analyses.
(4) Immunoprecipitant reactions.
(5) Ion exchange methods.
(6) Ion exclusion methods.

Description Of Invention

The major components for the preferred "light-switch" or "light indicator" of the present invention are similar to those disclosed in United States Patent No. 3,597,362. They include an oxalic ester, a hydroperoxide, a fluorescer (or fluorescent compound) and a diluent. Furthermore, in order to generate maximum intensity of light, the employment of an additional catalytic accelerator is sometimes necessary. The choice and the concentration and other parameters of a suitable catalytic accelerator is also described in United States Patent No. 3,704,231.

The present invention differs from the teaching of United States Patent No. 3,597,362 in that the fluorescent compound (or fluorescer) employed in this invention is covalently bonded to a biological material, such as immunoglobulin, enzymes, proteins, bacteria, and so on; or to an organic material, such as haptens or polymers; or to an inorganic material, such as glass, silica, ceramic, or the like. The organic and inorganic materials to which suitable fluorescer may be bonded can be in the form of particles, crystals, tubes, rods, plates, blocks and the like, or in solution. The fluorescent compound, or fluorescer, bonded to the above mentioned substances can then be utilized as a label in place of radioactive materials or an an indicator in place of color dye, for use in various well-known assays.

Especially suitable fluorescent compounds, or fluorescers, for use in the present invention are those which have a spectral emission falling between 260

millimicrons and 1,000 millimicrons. The structure of the fluorescent compounds or fluorescers useful in the present invention must possess one or more functional groups capable of reacting with those materials to be coupled to it. Examples of preferred functional groups are: alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino-, dihalo triazinyl-. Typical examples of suitable fluorescer derivatives are: 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, teteramethylrhodamine isothiocyanate, amino-pyrene, amino-anthracene, and similar compounds as will be familiar to those skilled in the art.

It has been observed that on binding a fluorescent compound fluorescer, to a solid material, the wavelength of emission of the bonded fluorescer shifts to either a longer or a shorter wavelength depending on the specific fluorescer employed.

We have also found that the length of "space arm", the ligand between the fluorescent compound and the material bonded to it, effects the emission wavelength of the bonded fluorescer.

The exact concentration of fluorescer derivative employed for binding is not critical providing that the immunological or enzymatic active conjugates produced therefrom have the desired activity, and that the intensity of light thus produced is visible, with or without

the help of instruments, and may be differentiated from the background.

The intensity of the light generated by the coupled fluorescer depends upon the structure of the fluorescer, the type of linkage between the fluorescer and the bonded materials, and the available functional groups of the anchored substance. In general, the intensity of the light produced by a fluorescer is not as great after coupling as it is when in free solution. It is also important that the fluorescer conjugate be stable in the presence of the chemiluminescent reaction.

The following examples are given to illustrate the various ways the fluorescer may be attached to another moiety by covalently bonding using an inorganic support for convenience, which is in no way intended to limit the scope of the invention described herein.

Examples I-V

In each of the Examples I-V the linkage attached to a controlled pore glass surface was synthesized to imitate the representative chemically active sites on a typical protein or biological conjugate. For example, amino-, carboxyl-, mercapto-, or hydroxyl-groups are representative of attachment sites.

A glass support is used so that the activity and specificity of the functional group is easily controlled, and to immobilize the fluorescer so that it may be readily separated from the free or unbound fluorescent compound in order that the fluorescent spectra may easily be recognized as distinct from the oxalate CL reagent.

The results of visual observation as to the color of the fluorescent glass, and color and intensity of emitted light for 1-aminopyrene covalently bonded to porous glass (CPG) (500Å pore size) fluorescer with various different linkages are set forth in the attached Table 7.

The methodology employed for preparing each fluorescer/glass sample was as follows:

Example I

Ten grams of porous glass of 500 (Å) (angstrom pore size) was treated with 100 ml 15% gamma-aminopropyl-trimethoxysilane in toluene and refluxed for at least 16 hours, then removed. The unbound silane was thoroughly washed with methanol, filtered and the glass air dried

before use. Approximately 25 milligrams 1-amin -pyrene was dissolved in dioxane (20 millimeter). To this solution about 153 milligrams of succinic anhydride was added. After two hours, 10 millimeter of 5 m mole N,N-dicyclohexyl-carbodiimide dioxane solution was added. 500 mg of this gamma-aminopoply-trimethoxysilane treated glass (from here on, aminopropyl-glass) as prepared above was added to dioxane solution. The slurry was then stirred for one hour and let stand overnight at room temperature. Continuous stirring is preferable. The excess pyrene-dioxane solution was decanted and the glass washed exhaustively with dioxane, methanol and acetone (15 ml of each wash and three times for each solvent). The wet pyrene coupled glass was filtered and allowed to air dry.

Example II

500 mg of the aminopropyl-glass prepared as stated in Example I was added to 25 ml of 10% thiophosgene in chlorofrom and the slurry was refluxed for 4 hours. The chloroform was decanted and then washed with chloroform, methanol, acetone (25 ml of each wash and three times for each solvent). The slurry was filtered and air dried. 30 milligrams of 1-aminopyrene was dissolved in 15 ml dioxane. To this solution, the dry isocyanato-glass was added and stirred for one hour and then allowed to stand at room temperature overnight. After the reaction was complete, aminopyrene dioxane solution was decanted and the pyrene coupled glass was washed in the same manner as stated in Example I.

Example III

500 mg of aminopropyl-glass, prepared as stated in Example I, was added to 10 ml of dioxane dissolved with 50 mg succinic anhydride. The slurry was allowed to stand overnight at room temperature preferably with continuous stirring. After the reaction was complete, the aminopropyl-glass, being converted to carboxy-glass, was washed in the same manner as stated in Example I. Approximately 23 mg 1-aminopyrene was dissolved in 1 ml of dioxane. To this solution, 58 mg of N-acetyhomocystein was dissolved. The solution was then kept 4 hours at room temperature. 50 mg of N,N-dicyclohexyl-carbodiimide was then added to it. At the same time, the prepared and dried carboxyl-glass was added to the solution for coupling. The reaction was allowed to stand at room temperature for 24 hours. Pyrene coupled glass was then washed and dried in the same manner as stated before.

Example IV

4 grams of aminopropyl-glass prepared from Example I was added to 10% p-nitrobenzoyl chloride with 1 ml of triethylamine in 50 ml chloroform solution. The slurry was stirred and refluxed for at least 8 hours. The resulting acylated glass was thoroughly washed with chloroform and let air dry. 0.1M of sodium dithionite (30 ml) was prepared and the acylated glass was added. The temperature was then raised to 40°C. The reaction was completed in one hour. The glass was washed thoroughly with warm water. The arylamino-glass thus prepared was ready to diazotize. 1 gm of arylamino-glass was added to 20 ml aqueous solution of 350 mg sodium nitrite and 0.2 ml 1N hydrochloric acid. The temperature was brought down to 4°C using an ice bath. The reaction

was allowed to continue for one hour. The acid solution was then decanted, the glass was thoroughly washed and the pH was adjusted to above 8.0. The filtered glass was then added to 10 ml of 20 mg aminopyrene dioxane solution. Reaction was complete in 8 hours at room temperature. The pyrene coupled glass was then washed in the same manner as in Example I.

Example V

One gram of 500 (Å) pore size porous glass treated with 10 ml 15% gamma-glycidoxypropyltrimethoxy-silane in toluene and refluxed for at least 16 hours, then washed the glass with acetone thoroughly and air dried. To 30 ml aqueous solution containing 1.5 mg/ml of m-sodium periodate, the silane treated glass (epoxy-glass) was added. The reaction was allowed to go on for 2 hours. Then the glass was washed with water thoroughly. 25 mg of 1-aminopyrene was dissolved in 30 ml dioxane. To this solution, the filtered wet cake glass was added. The slurry was stirred for one hour and then let stand overnight at room temperature. The pyrene coupled glass was washed in the same manner as stated in Example I.

TABLE 7 - EXAMPLES I-V

Effect of Different Linkages

Example No.	Type of Linkage	Structure	Color of Fluorescent Glass	Color the Light** Emits on Glass Particle	Intensity* Observed
I	Amido	Flr-N(H)-C(=O)--(CPG)	blue-green	bluish green	M
II	Thiourea	Flr-N(H)-C(=S)-N(H)--(CPG)	blue-green	bluish green	M
III	Thio-ester	Flr-S-C(=O)--(CPG)	grayish green	bluish green	W-M
IV	Diazo	Flr-N=N--(CPG	red-brown	red-brown	VW
V	Amide	Flr-N(H)-C(H)--(CPG)	as CPG	blue	M

* M = medium; W = weak; VW = very weak

** After addition of oxalate ester and hydrogen peroxide

Example VI

Different lengths of "space arm" for binding of the fluorescer were used to study the effect on the chemiluminescence quality of the resultant bonded fluorescer.

A long "space arm" of about 20 (Å) in length stretching out from a controlled glass pore surface was prepared as follows: 500 mg of carboxy-glass prepared as stated in Example III was activated by adding a 20 ml dioxane solution containing 200 mg of N,N-dicyclohexyl carbodiimide. The glass was stirred for 24 hours and then washed with dioxane and methanol. 20 ml of 200 mg hexamethylene diamine aqueous solution was prepared and cooled beforehand. The activated carboxy-glass was added to the cooled solution and stirred for five hours, then allowed to stand for 24 hours at 4°C. The glass was then washed thoroughly with water, methanol and dioxane. 20 ml dioxane containing 50 mg succinic anhydride was then added to the glass. This reaction was completed in 24 hours. The glass was subsequently washed thoroughly with methanol. 25 mg 1-aminopyrene was dissolved in 30 ml dioxane. To this solution 5 m mole N,N-dicylohexylcarbodiimide was added and dissolved prior to adding the prepared glass. The slurry was stirred for one hour and then let stand overnight at room temperature. After 24 hours reaction, pyrene coated glass was then washed in the same manner as in Example I.

Pyrene coated glass with a short "space arm" of about 10 (Å) in length was prepared as stated in Example I, as the control. The results of these two glasses is set forth in attached Table 8.

Table 8 - Example VI

Effect of "Space Arm" Length Between Glass Surface and The Fluorescer on Chemiluminescent Characteristics

Approximate Length of "Space Arm"	Color of Light	Intensity Observed*
(Example I) Control 10 (Å)	bluish-green	M
Example VI 20 (Å)	green	W-M

* M = medium; W = weak

Examples VII-IX

Porous glass having various pore sizes were coated with 1-aminopyrene to show the effect of pore size on the chemiluminescence. Three different porous glasses having 170 (Å) (angstrom), 500 (Å) and 3000 (Å) pore size, respectively, were coated with 1-aminopyrene in the same manner as stated in Example 1. The effect on the chemiluminescence is set forth in attached Table 9.

TABLE 9 - EXAMPLES VII-IX

Effect Of Pore Size On Chemiluminescence

Example No.	Pore Size (in angstrom)	Surface Area (in M^2/gm)	Color of Fluorescent Glass		Color of Light Emitted	Intensity Observed
			Before Coating	After Coating		
VII	170	110	clear	green	yellowish green	M
VIII	500	50	lt. blue	lt. blue	bluish green	W-M
IX	3000	10	white	white	blue	W

Examples X-XV

Several different fluorescers were coated on porous glass to study the effect of structure on color emission. 1-amino-pyrene and 2-amino-anthracene were coated on the porous glass (500 Å) in the same manner as described in Example I.

20 mg of 3,4,9,10-perylenetetracarboxylic dianhydride was added to 25 ml of dioxane, to this solution 25 mg of aminopropyl-glass was added and stirred for one hour before allowing to stand for another 6 hours at room temperature. The glass was then washed thoroughly with methanol or acetone, then filtered and air dried.

500 mg of aminopropyl-glass was added to 30 ml dioxane containing 50 mg succinic anhydride and stirred for one hour before being allowed to stand overnight at room temperature. The glass was then washed thoroughly with acetone, filtered and air dried. One part of 250 mg of such glass (carboxyl-glass) was added to 25 ml 0.01M potassium phosphate of pH=7.6 solution containing 20 mg of isothiocyanate fluorescein. Another part of 250 mg of carboxyl-glass was added to acetone/dioxane (50/50 by volume) solution containing 20 mg of 3-amino-phthalhydrazide). The two glass slurries were stirred for one hour and then allowed to stand at room temperature overnight. After the reaction was completed, the glass was washed with deionized water and acetone, respectively. Finally, they both were washed with acetone, then filtered and air dried.

300 mg of aminopropyl-glass prepar d as shown Example I was added to 50 ml 0.01M potassium phosphate of pH=7.6 solution containing 25 mg of O-phthalicdicarboxaldehyde. The glass slurry was stirred for one hour, then allowed to stand at room temperature for another 24 hours. The glass was then washed thoroughly with deionized water, acetone, then filtered and air dried.

The attached Table 10 sets forth the observed chemiluminescence characteristics of different fluorescers bonded to porous glass in an oxalate ester/peroxide system.

TABLE 10 - EXAMPLES X-XV

Comparison of Chemiluminescence of Different Fluorescers Free and Covalently Attached to Porous Glass of 500(A) Pore Size

Fluorescer	Example No.	Color of Fluorescent Glass	Color of Light - Bonded Fluorescer	Intensity of Light Observed	Color of Light - Free Fluorescer	Fluorescence U.V. Light
1-aminopyrene	X	blue green	bluish green	M	green	negative
2-amino-antracene	XI	lt. brown	violet	M	blue-violet	negative
3,4,9,10-perylene tetra-carboxylic dianhydride	XII	orange red	orange-red	M	none	orange
Fluorescein isothiocyanate	XIII	yellow	green	M	green	yellow
3-amino-phthal hydrazide (luminol)	XIV	lt. blue	bright blue	M	blue	blue
O-phthalicdicarboxaldehyde	XV	yellowish-brown	greenish-yellow	W-M	none	negative

Example XVI

Aminopyrene conjugate with antibody to Hepatitis B Surface Antigen coated on porous glass

30 mg commercially available antibody to Hepatitis B Surface Antigen coated porous glass was added to 5 ml of 0.01 m potassium phosphate of pH=7.6. 24 mg of 1-aminopyrene was dissolved in 2 ml dioxane. To this solution 45 mg of succinic anhydride was added and mixed for two hours. Approximately 95 mg of N,N-dicylohexyl-carbodiimide was dissolved in 1 ml of dioxane. The latter two solutions were mixed together and stirred for 30 minutes. Then 250 lamda of pyrene solution was transferred to the glass slurry solution. The slurry was stirred for two hours at room temperature and then allowed to stand at 4°C overnight. The glass was washed four times with 10 ml phosphate buffer (pH=7.6) each wash, and was given two additional t-butanol washes with 10 ml phosphate buffer each time before testing. If necessary, the slurry was washed until no light could be detected from the supernate of the slurry. Then the 1-amino-pyrene-antibody conjugate coated on the porous glass was tested by reacting with oxalate and peroxide. It was found that only the glass particle glowed in faint blue color.

Example XVII

Fluorescein isothiocyanate anti-human gamma-globulin conjugate was prepared as follows: 4 mg of fluorescein isothiocyanate thoroughly mixed in 10 ml 0.1 M potassium phosphate buffer of pH=9.0. 4 ml of anti-human gamma-globulin (protein concentration of 20 mg/ml) was

then added to the fluorescein phosphate solution. The mixture was continuously stirred for one hour at 4°C and allowed to stand at the same temperature for another 24 hours. Excess fluorescein was removed by extensive dialysis against 0.1 M potassium phosphate buffer of pH=7.2. During dialysis, 100 ml of buffer each time was used, and the buffer was changed every 2 hours for 5 times.

Gamma-globulin coated porous glass was prepared as follows: 50 mg of epoxy-glass (3000 (Å) pore size) was prepared in the same way as described in Example IV. 2.5 mg m-sodium periodate was dissolved in 5 ml of deionized water. Glass was then added to this solution and stirred at room temperature for 2 hours. The glass was washed thoroughly with deionized water and then with 10 ml 0.1 M potassium phosphate pH=9.0 buffer and kept for one hour. The glass was then filtered and was ready for coupling. 5 ml human gamma-globulin (protein concentration of 30 mg/ml) was diluted with 5 ml of 0.1 M, pH=9.0 phosphate buffer. The activated glass was then added to this solution and was stirred at 4°C for 2 hours before being allowed to stand overnight at the same temperature. After reaction was completed, the glass was washed extensively with 0.1 M potassium phosphate buffer of pH=7.2 and then filtered for immediate use.

30 mg of human gamma-globulin coated porous glass was added to 0.5 ml of fluorescein-antihuman gamma-globulin conjugate. The slurry was incubated on 24 cycles of agitation/settling (60/90 seconds ratio). Excess antibody solution was decanted and the glass was washed with 0.01 M potassium phosphate buffer of pH=7.2 until no light

was detected by testing the decanted buffer in oxalate/ peroxide system.

The glass was then washed with 5 ml t-butanol and excess butanol was withdrawn. Green color light was observed on glass particles upon addition of oxalate and peroxide.

Although the above examples illustrate various modifications of the present invention, other variations will suggest themselves to those skilled in the art in the light of the above disclosure. It is to be understood, therefore, that changes may be made in the particular embodiments described above which are within the full intended scope of the invention as defined in appended claims.

CLAIMS

1. A system for the detection of a biological analyte of interest which comprises contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an energy source which is capable of activating the fluorescer.

2. A system for the detection of a biological analyte of interest which comprises contacting a sample with a fluorescer which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an excess of an energy source which is capable of activating the fluorescer.

3. A method for the qualitative detection of a biological analyte of interest comprising:

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie;

(b) contacting the fluorescer labeled specie and the biological of interest;

(c) separating the fluorescer labeled specie/biological complex;

(d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and

(e) determining the presence or absence of chemiluminescent light emitted from the activated fluorescer.

4. A quantitative method for measuring the amount of a biological analyte of interest comprising;

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer material which is biologically compatible with such specie;

(b) contacting the fluorescer labeled specie and the biological of interest;

(c) separating the fluorescer labeled specie/biological complex;

(d) contacting the separated fluorescer labeled specie/biological complex of (c) with an energy source which is capable of activating the fluorescer label; and

(e) determining the amount of chemiluminescent light emitted from the activated fluorescer using appropriate instrumentation.

5. The method of claim 3 wherein the fluorescer of (a) is chemically conjugated to the immunological specie specific to the biological of interest.

6. The method of claim 5 wherein the chemical conjugation of the fluorescer material to the immunological specie specific to the biological of interest is carried out using known techniques in such a way as to prevent substantial biological damage to the attached specie.

7. The method of claim 3 wherein the fluorescer material utilized has a spectral emission of from about 260 millimicrons to about 1000 millimicrons.

8. The method of claim 3 wherein the fluorescer material utiliz d has a spectral emission above the light absorption wavelength of either the immunological specie specific to the biological of interest or the biological of interest and below the light absorption wavelength of any solvent system utilized.

9. The method of claim 3 wherein the fluorescer material utilized has a structure which possesses one or more functional groups capable of reacting with the immunological specie specific to the biological of interest without adversely affecting such specie.

10. The method of claim 3 wherein the fluorescer material utilized has a structure which possesses one or more functional groups selected from the group comprising alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl-.

11. The method of claim 3 wherein the fluorescer material utilized is selected from the group comprising 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothio-cyanate, teteramethyl-rhodamine isothiocyanate, amino-pyrene, amino-anthracene, and the like.

12. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is present in excess of the amount required to activate all of the fluorescer labeled specie.

13. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is any source which is capable of activating the particular flourescer selected to be compatible with the labeled specie.

14. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is the peroxyoxylate reaction.

15. A method according to claim 13 wherein the energy source is a reaction selected from the group comprising 2-napthol-3,6,8-trisulfonic acid, 2-carboxyphenyl, 2-carboxy-6-hydroxyphenol, 1,4-dihydroxy-9, 10-diphenyl-anthracene, 2-napthol, luminol, lophine, pyrogallol and luciferin reactions.

16. A method according to claim 13 wherein the energy source is derived from ozone, an electrogenerated species, or a mechanically generated species.

17. A method according to claim 3 which is carried out utilizing solid phase analytical techniques.

18. A method according to claim 3 which is carried out utilizing a sandwich technique.

19. A method according to claim 3 which is carried out utilizing homogeneous analytical techniques.

20. A method according to claim 3 which is carried out utilizing heterogeneous analytical techniques.

21. A method according to claim 3 which is carried out utilizing competitive binding techniques.

22. A method according to claim 3 which is carried out utilizing quenching techniques.

23. A method according to claim 3 which is carried out utilizing immuno-precipitant reaction techniques.

24. A method according to claim 3 which is carried out utilizing ion exchange techniques.

25. A method according to claim 3 which is carried out utilizing ion exclusion techniques.

26. A method according to claim 3 which is carried out utilizing masking techniques.

27. The method of claim 4 wherein the fluorescer of (a) is chemically conjugated to the immunological specie specific to the biological of interest.

28. The method of claim 4 wherein the chemical conjugation of the fluorescer material to the immunological specie specific to the biological of interest is carried out using known techniques in such a way as to prevent substantial biological damage to the attached specie.

29. The method of claim 4 wherein the fluorescer material utilized has a spectral emission of from about 260 millimicrons to about 1000 millimicrons.

30. The method of claim 4 wherein the fluorescer material utilized has a spectral emission above the light absorption wavelength of either the immunological specie specific to the biological of interest or the biological of interest and below the light absorption wavelength of any solvent system utilized.

31. The method of claim 4 wherein the fluorescer material utilized has a structure which possesses one or more functional groups capable of reacting with the immunological specie specific to the biological of interest without adversely affecting such specie.

32. The method of claim 4 wherein the fluorescer material utilized has a structure which possesses one or more functional groups selected from the group comprising alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl-.

33. The method f claim 4 wherein the fluorescer material utilized is selected from the group comprising 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, teteramethylrhodamine isothiocyanate, amino-pyrene, amino-anthracene, and the like.

34. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is present in excess of the amount required to activate all of the fluorescer labeled specie.

35. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is any source which is capable of activating the particular fluorescer selected to be compatible with the labeled specie.

36. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer labeled specie/biological complex is the peroxyoxylate reaction.

37. A method according to claim 35 wherein the energy source is a reaction selected from the group comprising 2-napthol-3,6,8-trisulfonic acid, 2-carboxyphenyl, 2-carboxy-6-hydroxyphenol, 1,4-dihydroxy-9,10-diphenylanthracene, 2-napthol, luminol, lophine, pyrogallol, luciferin reactions.

38. A method according to claim 35 wherein the energy source is derived from ozone, an electrogenerated species or a mechanically generated species.

39. A method according to claim 4 which is carried out utilizing solid phase analytical techniques.

40. A method according to claim 4 which is carried out utilizing homogeneous analytical assay techniques.

41. A method according to claim 4 which is carried out utilizing heterogeneous analytical assay techniques.

42. A method according to claim 4 which is carried out utilizing competitive binding techniques.

43. A method according to claim 4 which is carried out utilizing quenching analyses techniques.

44. A method according to claim 4 which is carried out utilizing immuno-precipitant reaction techniques.

45. A method according to claim 4 which is carried out utilizing ion exchange techniques.

46. A method according to claim 4 which is carried out utilizing ion exclusion techniques.

47. A method according to claim 4 which is carried out utilizing masking techniques.

48. A fluorescer composition useful in the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer having a chemical structure which possesses one or more functional groups capable of chemical reaction with the immunological specie without the adverse effect on the specificity of such specie to the biological of interest.

49. A fluorescer composition useful in the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer having a chemical structure which posseses one or more functional groups selected from the group comprising alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl.

50. A fluorescer composition useful in the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer selected from the group comprising 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, teteramethylrhodamine isothiocyanate, amino-pyrene, amino-anthracene, and the like.

51. A conjugated fluorescer/immun logical specie composition useful in the detection of a biological of interest which has been formed via reacting an immunological specie with a fluorescer having a chemical structure which possesses one or more functional groups capable of chemical reaction with the immunological specie without adverse effect on the specificity of such specie to the biological of interest.

52. A conjugated fluorescer/immunological specie composition useful in the detection of a biological of interest which has been formed via reacting an immunological specie with a fluorescer having a chemical structure which possesses one or more functional groups selected from the group comprising/ alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino and dihalo triazinyl-.

53. A conjugated fluorescer/immunological specie composition useful in the detection of a biological of interest which has been formed via reacting an immunological specie with a fluorescer selected from the group comprising 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, amino-pyrene, amino-anthracene, and the like.

AMENDED CLAIMS
(received by the International Bureau on 27 May 1981 (27.05.81))

1. A system for the detection of a biological analyte of interest which comprises a fluorescer-catalyst which has been conjugated to an immunological specie specific to the biological analyte of interest, in tne presence of an energy source other than electromagnetic radiation capable of activating the fluorescer-catalyst.

2. A system for the detection of a biological analyte of interest which comprises a fluorescer-catalyst which has been conjugated to an immunological specie specific to the biological analyte of interest, in the presence of an excess of an energy source other than electromagnetic radiation which is capable of activating the fluorescer-catalyst.

3. A method for the qualitative detection of a biological analyte of interest comprising:

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer-catalyst material which is biologically compatible with such specie;

(b) contacting the fluorescer-catalyst labeled specie and the biological of interest;

(c) separating the fluorescer-catalyst labeled specie/biological complex;

(d) contacting the separated fluorescer-catalyst labeled specie/biological complex of (c) with an energy source other than electromagnetic radiation which is capable of activating the fluorescer label; and

(e) determining the presence or absence of light emitted from the activated fluorescer-catalyst.

4. A quantitative method for measuring the amount of a biological analyte of interest comprising:

(a) labeling an immunological specie specific to the analyte of interest with a fluorescer-catalyst material which is biologically compatible with such specie;

(b) contacting the fluorescer-catalyst labeled specie and the biological of interest;

(c) separating the fluorescer-catalyst labeled specie/biological complex;

(d) contacting the separated fluorescer-catalyst labeled specie/biological complex of (c) with an energy source other than electromagnetic radiation which is capable of activating the fluorescer label; and

(e) determining the amount of light emitted from the activated fluorescer-catalyst.

5. The method of claim 3 wherein the fluorescer-catalyst of (a) is chemically conjugated to the immunological specie specific to the biological of interest.

6. The method of claim 5 wherein the chemical conjugation of the fluorescer-catalyst material to the immunological specie specific to the biological of interest is carried out using known techniques in such a way as to prevent substantial biological damage to the attached specie.

7. The method of claim 3 wherein the fluorescer-catalyst material utilized has a spectral emission of from about 260 millimicrons to about 1000 millimicrons.

8. The method of claim 3 wherein the fluorescer-catalyst material utilized has a spectral emission above the light absorption wavelength of either the immunological specie specific to the biological of interest or the biological of interest and below the light absorption wavelength of any solvent system utilized.

9. The method of claim 3 wherein the fluorescer-catalyst material utilized has a structure which possesses one or more functional groups capable of reacting with the immunological specie specific to the biological of interest without adversely affecting such specie.

10. The method of claim 3 wherein the fluorescer-catalyst material utilized has a structure which possesses one or more functional groups selected from the group consisting of alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl.

11. The method of claim 3 wherein the fluorescer-catalyst material utilized is selected from the group consisting of 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothio-cyanate, teteramethyl-

rhodamine isothiocyanate, amino-pyrene and amino-anthracene.

12. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is present in excess of the amount required to activate all of the fluorescer-catalyst labeled specie.

13. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is any source other than electromagnetic radiation which is capable of activating the particular fluorescer-catalyst selected to be compatible with the labeled specie.

14. The method of claim 3 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is the peroxyoxylate reaction.

15. A method according to claim 13 wherein the energy source is a chemical reaction selected from the group consisting of 2-napthol-3,6,8-trisulfonic acid, 2-carboxyphenyl, 2-carboxy-6-hydroxyphenol, 1,4-dihydroxy-9, 10-diphenyl-anthracene, 2-napthol, luminol, lophine, pyrogallol and luciferin reactions.

16. A method according to claim 13 wherein the energy source is derived from a chemical reaction, ozone, an electric current, an electrochemical reaction, or a mechanically generated species.

17. A method according to claim 3 which is carried out utilizing solid phase analytical techniques.

18. A method according to claim 3 which is carried out utilizing a sandwich technique.

19. A method according to claim 3 which is carried out utilizing heterogeneous analytical techniques.

20. A method according to claim 3 which is carried out utilizing heterogeneous competitive binding techniques.

21. A method according to claim 3 which is carried out without separating the fluorescer-catalyst labeled specie/biological complex utilizing quenching analyses techniques.

22. A method according to claim 3 which is carried out utilizing immuno-precipitant reaction techniques.

23. A method according to claim 3 which is carried out utilizing ion exchange techniques.

24. A method according to claim 3 which is carried out utilizing ion exclusion techniques.

25. A method according to claim 3 which is carried out utilizing masking techniques.

26. The method of claim 4 wherein the fluorescer-catalyst of (a) is chemically conjugated to the immunological specie specific to the biological of interest.

27. The method of claim 4 wherein the chemical conjugation of the fluorescer-catalyst material to the immunological specie specific to the biological of interest is carried out in such a way as to prevent substantial biological damage to the attached specie.

28. The method of claim 4 wherein the fluorescer-catalyst material utilized has a spectral emission of from about 260 millimicrons to about 1000 millimicrons.

29. The method of claim 4 wherein the fluorescer-catalyst material utilized has a spectral emission about the light absorption wavelength of either the immunological specie specific to the biological of interest or the biological of interest and below the light absorption wavelength of any solvent system utilized.

30. The method of claim 4 wherein the fluorescer-catalyst material utilized has a structure which possesses one or more functional groups capable of reacting with the immunological specie specific to the biological of interest without adversely affecting such specie.

31. The method of claim 4 wherein the fluorescer-catalyst material utilized has a structure which possesses one or more functional groups selected from the group consisting of alkylamino-, arylamino-, isocyano-, cyano-,

isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl.

32. The method of claim 4 wherein the fluorescer-catalyst material utilized is selected from the group consisting of 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, teteramethyl-rohodamine isothiocyanate, amino-pyrene, and amino-anthracene.

33. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is present in excess of the amount required to activate all of the fluorescer-catalyst labeled specie.

34. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is any source other than electromagnetic radiation which is capable of activating the particular fluorescer-catalyst selected to be compatible with the labeled specie.

35. The method of claim 4 wherein the energy source of (d) which is contacted with the separated fluorescer-catalyst labeled specie/biological complex is the peroxyoxylate reaction.

36. A method according to claim 35 wherein the energy source is a chemical reaction selected from the group consisting of 2-napthol-3,6,8-trisulfonic acid, 2-carboxyphenyl, 2-carboxy-6-hydroxyphenol, 1,4-dihydroxy-9,10-diphenylanthracene, 2-napthol, luminol, lophine, pyrogallol, and luciferin reactions.

37. A method according to claim 35 wherein the energy source is derived from a chemical reaction, ozone, an electrical current, an electrochemical reaction, or a mechanically generated species.

38. A method according to claim 4 which is carried out utilizing solid phase analytical techniques.

39. A method according to claim 4 which is carried out utilizing heterogeneous analytical assay techniques.

40. A method according to claim 4 which is carried out utilizing heterogeneous competitive binding techniques.

41. A method according to claim 4 which is carried out without separating the fluorescer-catalyst labeled specie/biological complex utilizing quenching analyses techniques.

42. A method according to claim 4 which is carried out utilizing immuno-precipitant reaction techniques.

43. A method according to claim 4 which is carried out utilizing ion exchange techniques.

44. A method according to claim 4 which is carried out utilizing ion exclusion techniques.

45. A method according to claim 4 which is carried out utilizing masking techniques.

46. A fluorescer-catalyst composition useful in the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer-catalyst having a chemical structure which possesses one or more functional groups capable of chemical reaction with the immunological specie without the adverse effect on the specificity of such specie to the biological of interest.

47. A fluorescer-catalyst composition useful in the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer-catalyst having a chemical structure which possesses one or more functional groups selected from the group consisting of alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole-, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino- and dihalo triazinyl.

48. A fluorescer-catalyst composition useful in

the labeling of an immunological specie specific to and for the detection of a biological of interest, such fluorescer-catalyst selected from the group consisting of 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, teteramethylrhodamine isothiocyanate, amino-pyrene, and amino-anthracene.

49. A conjugated fluorescer-catalyst/immunological specie composition useful in the detection of a biological of interest which has been formed via reacting an immunological specie with a fluorescer-catalyst having a chemical structure which possesses one or more functional groups capable of chemical reaction with the immunological specie without adverse effect on the specificity of such specie to the biological of interest.

50. A conjugated fluorescer-catalyst/immunological specie composition useful in the detection of a biological of interest which has been formed via reacting an immunological specie with a fluorescer-catalyst having a chemical structure which possesses one or more functional groups selected from the group consisting of alkylamino-, arylamino-, isocyano-, cyano-, isothiocyano-, thiocyano-, carboxy-, hydroxy-, mercapto-, phenol-, imidiazole, aldehyde-, epoxy-, thionyl halide-, sulfonyl halide-, nitrobenzoyl halide-, carbonyl halide-, triazo-, succinimido-, anhydride-, haloacetate-, hydrazino and dihalo triazinyl-.

51. A conjugated fluorescer-catalyst/immunological specie composition useful in tne detection of a biological of interest which has been formed via reacting an immuno-

logical specie with a fluorescer-catalyst selected from the group consisting of 3,4,9,10 perylene tetracarboxylic dianhydride, amino-chrysene, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, amino-pyrene, and amino-anthracene.

EDITORIAL NOTE

The applicant failed to renumber the amended claims in accordance with Section 205 of the Administrative Instructions.

In the absence of any specific indication from the applicant as to the correspondence between original and amended claims, these claims are published as filed and as amended.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US80/01485

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] G01N 33/54

U.S. CL. 424/8,12; 23/230B

II. FIELDS SEARCHED

Minimum Documentation Searched [4]	
Classification System	Classification Symbols
U.S.	424/8,12; 23/230B

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US,A, 3,935,074, PUBLISHED 27 JANUARY 1976, RUBENSTEIN ET AL.	1,2,3-13,17-34 39-37,48-53
X	US,A, 4,011,219, PUBLISHED 08 MARCH 1977, NISHII ET AL.	13-16,36-38
A	US,A, 4,018,884, PUBLISHED 19 APRIL 1977, CLEELAND JR. ET AL.	---
X	US,A, 4,020,151, PUBLISHED 26 APRIL 1977, BOLZ ET AL.	1,2,3-13,17-34 39-37,48-53
X	US,A, 4,104,029, PUBLISHED 01 AUGUST 1978, MAIER JR..	13-16,36-38
A	US,A, 4,153,675, PUBLISHED 08 MAY 1979, KLEINERMAN.	---
A	US,A, 4,160,818, PUBLISHED 10 JULY 1979, SMITH ET AL.	---
X	US,A, 4,169,137, PUBLISHED 25 SEPTEMBER 1979, HIRSHFELD ET AL.	1,2,3-13,17-34 39-37,48-53
P	US,A, 4,181,650, PUBLISHED 01 JANUARY 1980, MAIER JR..	---
A,P	US,A, 4,199,559, PUBLISHED 22 APRIL 1980, ULLMAN ET AL.	---

* Special categories of cited documents: [15]

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 MARCH 1981	27 MAR 1981
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	SIDNEY MARANTZ

Form PCT/ISA/210 (second sheet) (October 1977)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,P	US,A, 4,201,763, PUBLISHED 06 MAY 1980, MONTHONY ET AL.	1,2,3-13,17-34 39-37,48-53
X,P	US,A, 4,220,450, PUBLISHED 02 SEPTEMBER 1980, MAGGIO.	13-16,36-38
X,E,	US,A, 4,238,195, PUBLISHED 09 DECEMBER 1980, BOGUSLASKI ET AL.	13-16,36-38

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.